were produced when our pTrans2 was replaced with published pACG2 and pXX2 while using the same pHelper and pCis-EGFP (FIG. **8**A-C) implies that the configuration of pTrans may also play a role in rAAV vector production yields.

[0206] TN1 and sodium butyrate were used throughout our optimization process because higher capsid protein expression was achieved by using these additives in our previous optimization (Zhao, H et al., Cost effective and facile method of rAAV production in suspension-adapted HEK293 cells, Mol. Ther. 19, Supplement 1:S257 (2011)) and it was previously reported that TN1 addition increased rAAV2-EGFP production by 30% (Hildinger, M. et al., High-titer, serum-free production of adeno-associated virus vectors by polyethyleneimine-mediated plasmid transfection in mammalian suspension cells, Biotechnol. Lett. 29:1713-21 (2007)). The roles of TN1 and sodium butyrate in the DOE optimized protocol were examined again and the results indicated that TN1 played a critical role in rAAV vector production. In contrast, sodium butyrate was not necessary (FIG. **11**A-C) although that was not the case in our OFAT-optimized protocol (data not shown). The underlying mechanism of TN1 in rAAV vector production is not clear. However, TN1 has been reported to increase both the mRNA and recombinant protein expression levels, suggesting it might act at both transcriptional and translational levels (Pham, P L et al., Transient gene expression in HEK293 cells: peptone addition posttransfection improves recombinant protein synthesis. Biotechnol Bioeng. 90:332-44 (2005)).

[0207] In an attempt to compare rAAV vector production yields of both DOE-improved and published OFAT-optimized protocols, Durocher's protocol was chosen for comparison due to its simplicity and similarity to our procedure (Durocher, Y et al., Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. J Virol Methods. 144:32-40 (2007)). Unexpectedly, much lower amounts of rAAV2 and rAAV2/8 vectors were produced using Durocher's protocol (<5%) as compared to the DOE-improved protocol. This implies that optimization using the DOE method is superior to OFAT and that pTrans plays an important role in efficient rAAV vector production.

[0208] When yields of rAAV vector production for a particular GOI are much lower than that of average production yields, these poor yields can be dramatically increased by further decreasing the DOE-optimized amount of pCis plasmid. FIG. **12**A shows that the yields of some rAAV vectors (rAAV8-Kcnj14 and -Paqr9) can be very low as compared to other rAAV vectors produced under the same conditions. In the experiment, rAAV vectors were produced in 1-liter cultures by triple transfection using DOE-optimized conditions as described in Example 2 above and pHelper, pTrans2/8 and pCis encoding the GOIs. After 72 hours, transfected cells and conditioned media containing rAAV vectors were harvested and AAV cap expression was analyzed by immunoblot analysis using anti-VP antibody (Fitzgerald Industries International, Inc., Acton, Mass., cat. #10R-A114a). The amino acid sequences of the C-terminal ends of AAV VP1, VP2, and VP3 are identical; the anti-VP antibody recognizes the peptide sequence of the C-terminus (726-733) that is common for all 3 VPs. FIGS. **12**B and **12**C show that the AAV cap expression (B) and GC (genome copies, C) of rAAV8-Kcnj14 significantly increased with the decreased amount of pCis-Kcnj14. In the experiment, rAAV8-Kcnj14 was generated in 20-mL cultures using DOE-optimized conditions with two times serial dilutions of pCis-Kcnj14 from 1 (DOE-optimized amount) to 256 times (1/256 of DOE-optimized amount). After harvest, VPs and GCs were analyzed by immunoblot (FIG. **12**B) and CyQuant fluorescent method (FIG. **12**C) after rAAV vector purification, respectively; FIG. **12**D and FIG. **12**E show that yields of rAAV8-Paqr9 and rAAV8-Kcnj14 dramatically increased using 1/32 or 1/64 of the amount of DOE-optimized pCis. These two rAAVs were produced in 1-liter culture using DOE-optimized conditions (1) and reduced amount of pCis (1/32 or 1/64). The produced rAAV8 vectors were purified with AVB-Sepharose and quantified by CyQuant fluorescent method.

[0209] It appears that the production yields of rAAVvectors may significantly differ for different pCis-GOIs, though the DOE-optimized methods are applied to the rAAV vector production under the same conditions. As shown in FIG. **12**A, although these eight different rAAV vectors were produced using the DOE-improved protocol, the amount of AAV cap expression (therefore, it correlated with the yield of rAAV vector production) co-transfected with some genes such as Kcnj14 Paqr9, were very low as compared to other genes (FIG. **12**A). Since GOI can be expressed by the pCis-GOI plasmid, we speculated that the decreased rAAV vector production could be caused by the gene product of GOI. Thus, the pCis-Kcnj14 was serially diluted from 1 (amount of DOE-improved protocol) to 256 times for rAAV vector production in 20-mL culture, and the AAV cap expression and rAAV GCs were monitored. FIG. **12**B and FIG. **12**C show that, following the dilution of pCis plasmid, both AAV cap protein expression and the yield of rAAV-Kcnj14 significantly increased, which was further confirmed by large scale (1-liter) production of these rAAVs (FIG. **12**D and FIG. **12**E). These results show that yields of rAAV8-Paqr9 and rAAV8-Kcnj14 were increased dramatically by using 1/32 or 1/64 amounts of pCis. GOI expression is a byproduct of rAAV vector production and not necessary for generating rAAV vectors. Furthermore, GOI products could affect rAAV vector production, depending on the function of the GOI. The results showed here indicate that, in some cases, GOI expression can be destructive for rAAV vector production. Thus, reduction of pCis plasmid and, therefore, reduction of GOI expression can be beneficial to the yield of rAAV vector production.

SEQUENCE LISTING

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